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Transcriptional Regulation of the Astrocytic Excitatory Amino Acid Transporter 1 (EAAT1) via NF-κB and Yin Yang 1 (YY1)*

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Background: The mechanism for transcriptional regulation of EAAT1 remains to be elucidated.
Results: EGF-activated NF-κB is a positive regulator of EAAT1, whereas manganese-activated YY1, with HDACs acting as co-repressors, is a negative regulator.
Conclusion: NF-κB and YY1 are two critical transcriptional regulators of EAAT1.
Significance: Identifying the molecular targets of EAAT1 regulation is crucial to develop therapeutics against neurological disorders associated with impairment of EAAT1.

Astrocytic glutamate transporter excitatory amino acid transporter (EAAT) 1, also known as glutamate aspartate transporter (GLAST) in rodents, is one of two glial glutamate transporters that are responsible for removing excess glutamate from synaptic clefts to prevent excitotoxic neuronal death. Despite its important role in neurophysiological functions, the molecular mechanisms of EAAT1 regulation at the transcriptional level remain to be established. Here, we report that NF-κB is a main positive transcription factor for EAAT1, supported by the following: 1) EAAT1 contains two consensus sites for NF-κB, 2) mutation of NF-κB binding sites decreased EAAT1 promoter activity, and 3) activation of NF-κB increased, whereas inhibition of NF-κB decreased EAAT1 promoter activity and mRNA/protein levels. EGF increased EAAT1 mRNA/protein levels and glutamate uptake via NF-κB. The transcription factor yin yang 1 (YY1) plays a role as a critical negative regulator of EAAT1, supported by the following: 1) the EAAT1 promoter contains multiple consensus sites for YY1, 2) overexpression of YY1 decreased EAAT1 promoter activity and mRNA/protein levels, and 3) knockdown of YY1 increased EAAT1 promoter activity and mRNA/protein levels. Manganese decreased EAAT1 expression via YY1. Epigenetic modifiers histone deacetylases (HDACs) served as co-repressors of YY1 to further decrease EAAT1 promoter activity, whereas inhibition of HDACs reversed manganese-induced decrease of EAAT1 expression. Taken together, our findings suggest that NF-κB is a critical positive regulator of EAAT1, mediating the stimulatory effects of EGF, whereas YY1 is a negative regulator of EAAT1 with HDACs as co-repressors, mediating the inhibitory effects of manganese on EAAT1 regulation.

Glutamate is the principal excitatory neurotransmitter in the CNS, playing critical roles in learning, cognition, and memory (1), but excessive levels of glutamate in the synaptic clefts induce excitotoxic neuronal death by overstimulation of post synaptic glutamate receptors. Thus, optimal levels of glutamate must be kept in the synaptic cleft by continuous uptake into adjacent astrocytes via astrocytic glutamate transporters. Among the five subtypes of glutamate transporters, excitatory amino acid transporter (EAAT)[2] 1 and 2 in humans, also known as glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) in rodents, are responsible for the majority of glutamate clearance and are predominantly expressed in astrocytes (2, 3). Dysregulation of these astrocytic glutamate transporters leads to accumulation of glutamate in the synaptic clefts, which overstimulates glutamate receptors, resulting in calcium overload in postsynaptic neurons and ultimately excitotoxic neuronal death. In particular, multiple neurological diseases including Alzheimer disease (AD) (4, 5), ataxia (6, 7), alcoholism (8), and traumatic brain injuries (9) are associated with dysregulation of GLAST/EAAT1. The decreased expression of EAAT1 has also been noted in ophthalmic disorders, including glaucoma (10), and a recent study reported that arundic acid-induced increase in EAAT1 expression and function rescues retinal ganglion cell death in a rodent model of glaucoma (11). Animal studies also reveal that inhibition of EAAT1 increases extracellular glutamate with subsequent excitotoxic neuronal death (12). EAAT1 deficiency in rodents induces a lack of motor coordination and increases susceptibility to cerebellar injury (13), epilepsy (14), and ischemic retinal damage (15, 16).

Despite the critical roles of EAAT1 in CNS neurophysiology and neuropathology, the molecular mechanisms involved in the

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[2] The abbreviations used are: EAAT, excitatory amino acid transporter; GLAST, glutamate aspartate transporter; YY1, yin yang 1; HDAC, histone deacetylase; VPA, valproic acid; TSA, trichostatin A; AD, Alzheimer disease; PDTC, pyrrolidine dithiocarbamate; QNZ, 6-amino-4-(4-phenoxy-phenylethylamino)quinazoline; qPCR, quantitative real time PCR; Co-IP, co-immunoprecipitation; DAPA, DNA affinity purification assay; ANOVA, analysis of variance; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

regulation of EAAT1 at the transcriptional level are not completely understood. The EAAT1 promoter is stimulated by growth factors such as EGF and TGF-α, leading to enhancement of EAAT1 mRNA expression (17, 18), as well as protein levels (19, 20). Estrogen and selective estrogen receptor modulators, such as tamoxifen and raloxifene, also enhance EAAT1 mRNA and protein levels (21, 22). The transcription factor NF-κB has been reported to be a critical positive regulator of GLT-1/EAAT2 and mediates the stimulatory effects of various modulators such as ceftriaxone (23), EGF (24, 25), tamoxifen, and raloxifene (21, 26) on EAAT2 expression. Earlier studies have also suggested that transcriptional regulation of EAAT1 might be different from that of EAAT2 because the mTOR-PI3k/Akt signaling cascade induced only EAAT2 expression (27, 28). Some studies show that NF-κB might also play a critical role in the regulation of EAAT1 expression induced by EGF, TGF-α, and raloxifene in rat primary astrocytes (19, 21). However, the precise relationship between NF-κB and EAAT1 has yet to be characterized.

On the other hand, the transcription factor Yin Yang 1 (YY1) is involved in the negative regulation of chick EAAT1 promoter that contains multiple YY1 binding sites (29, 30). YY1 is an ubiquitously expressed multifunctional protein that can activate or repress gene expression depending on the cellular context (31), and therefore its role varies from cell proliferation and differentiation to apoptosis in various cell types (32). It also plays crucial roles in the nervous system, participating in neural development, neuronal function, developmental myelination, and neurological diseases (33). Importantly, YY1 is a repressor of EAAT2 (34, 35), but its role in the regulation of EAAT1 requires further characterization. It has been reported that excess glutamate induces YY1 binding to the EAAT1 promoter, resulting in decreased glutamate uptake and EAAT1 mRNA levels in chick Bergmann glia cells (30). The same group showed that the anti-epileptic drug valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, increases EAAT1 promoter activity, mRNA/protein levels, and uptake activity in chick Bergmann glia cells while decreasing YY1 binding to the EAAT1 promoter (29), indicating a role for YY1 in repressing EAAT1. TNF-α decreases EAAT1 promoter activity, mRNA, and protein levels (18, 21). Manganese, an environmental toxin that causes Parkinson disease-like pathological signs and symptoms, referred to as manganism, decreases EAAT1 expression and function in astrocytes (21, 22, 36). However, the molecular mechanisms involved in manganese-induced repression of EAAT1 at the transcriptional level remain to be established.

HDACs are a class of enzymes that remove acetyl groups from L-lysine amino acid on proteins. HDACs play a key role in homeostasis of protein acetylation in histones and non-histone proteins to regulate fundamental cellular activities such as transcription (37). A wide range of neurological diseases such as AD, Parkinson disease, Huntington disease, ischemic stroke, and schizophrenia are associated with imbalances between protein acetylation and deacetylation that result in transcriptional dysfunction (reviewed in Ref. 38). Dysregulation of HDAC functions may be involved in these disease processes because inhibition of HDACs exerted neuroprotective effects in various animal disease models, including stroke (39–41), Huntington disease (42), amyotrophic lateral sclerosis (43), AD (44), and Parkinson disease (45).

In addition to VPA, other HDAC inhibitors such as trichostatin A (TSA) and sodium butyrate (NaB) also reduce YY1 bindings to DNA, indicating that inhibition of YY1 binding to DNA is universal for HDAC inhibitors in YY1-mediated repression of gene transcription. Moreover, VPA and TSA have also been shown to increase EAAT1 expression by increasing acetylation of histones H3 and H4 (29, 46). These findings indicate that HDAC inhibitors enhance EAAT1 expression via increased acetylation of both histone and non-histone protein molecules. In addition, inhibition of HDAC protects against glutamate excitotoxicity-induced neurological disorders in various *in vitro* as well as *in vivo* animal model studies (47–49). Because impaired EAAT1 function directly leads to glutamate excitotoxic neuronal injury associated with numerous neurological disorders, inhibition of HDACs is likely exerting neuroprotective effects through enhanced EAAT1 function via inhibition of transcriptional activities of genes regulated by HDACs.

In the present study, we investigated the mechanism of positive as well as negative regulation of EAAT1 using EGF as a stimulator and manganese as a repressor at the transcriptional level in rat primary astrocytes and human astrocytic H4 cells. Our results establish that NF-κB is a key positive regulator in mediating the stimulatory effects of EGF, whereas YY1 is a critical negative regulator in mediating the inhibitory effects of manganese on EAAT1 expression.

Experimental Procedures

Materials—All cell culture media and reagents were purchased from Invitrogen unless stated otherwise. Luciferase reporter assay kit was obtained from Promega (Madison, WI). Manganese chloride ($MnCl_2$), pyrrolidine dithiocarbamate (PDTC), 6-amino-4-(4-phenoxy-phenylethylamino)quinazoline (QNZ), VPA, TSA, and NaB were obtained from Sigma-Aldrich. Romidepsin (FK228) and suberoyl-anilide hydroxamic acid were from Selleck Chemicals (Houston, TX). EAAT1 antibody was from Abcam (Cambridge, MA). YY1, NF-κB (p65 and p50), IκBα, β-actin, and histone H3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human YY1 shRNA, control shRNA, and copGFP control lentiviral particles, Polybrene, and puromycin dihydrochloride were also from Santa Cruz. L-[^{3}H]Glutamate was purchased from PerkinElmer Life Sciences.

Astrocyte Culture—The primary cultures of astrocytes were prepared from cortices of 1-day-old Sprague-Dawley rats as described previously (50). Briefly, cerebral cortices were taken from the brain of rat pups and digested with Dispase and DNase I. The tissue digest was spun at 1000 rpm for 5 min, and the cell pellet was dissolved in growth medium. Astrocytes were plated at a density of 1×10^5 cells/ml in 150-cm^2 tissue culture flasks. The fresh media was replenished after 24 h of initial plating, and the cultures were maintained at 37 °C in a 95% air, 5% CO_2 incubator for 3 weeks in minimum essential medium supplemented with 10% horse serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. All experiments were carried out 3-weeks post isolation of astrocytes, and cells were plated in 24-well plates for luciferase assay and in 6-well plates for

mRNA/protein analysis. The purity of astrocyte culture was confirmed with >95% positive staining for the astrocyte-specific marker glial fibrillary acidic protein. Human astrocytic H4 cell line was obtained from ATCC (HTB-148) and grown in DMEM with 10% fetal bovine serum and 1% penicillin and streptomycin. The cultures were maintained at 37 °C in a 95% air, 5% CO_2 incubator.

Mutagenesis—pGL3 EAAT1 plasmid vector (a generous gift from Dr. Volsky, Columbia University, New York, NY) was used as an original template (containing 2072 bp from +99 to −1973) (18) to mutate NF-κB consensus binding sites (−116 and −538 position) on GLAST/EAAT1 promoter. These putative consensus sites on the EAAT1 promoter were confirmed by the web-based program Promo (ALGGEN). The mutation was performed using a QuikChange site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA). The primer sets used were as follows: for −116 mutation, 5′-CCA GAA ACC TCG AGG TTT CTC TCT CCT CCC TGT G-3′ (forward) and 5′-CAC AGG GAG GAG AGA GAA ACC TCG AGG TTT CTG G-3′ (reverse); and for −538 mutation, 5′-TGC TGA AAT AGA GAC ATG TCT CTA ACT TTA GAC-3′ (forward) and 5′-GTC TAA AGT TAG AGA CAT GTC TCT ATT TCA GCA-3′ (reverse). The mutant clones were confirmed by sequencing.

Luciferase Assay—EAAT1 promoter activity was determined by transfecting cells with pGL3 EAAT1 luciferase plasmid vector containing the EAAT1 promoter sequences from +99 to −1973 (18). Before transfection, the cells were grown for 2–3 days, and the transfection was done with Lipofectamine 2000. In some cases, the cells were treated with the designated compounds in Opti-MEM after transfection. Luciferase activity was measured with the Bright-Glo luciferase kit (Promega, Madison, WI) according to the manufacturer's instructions and normalized to the protein content as determined by BCA assay (Thermo Fisher Scientific, Rockford, IL).

Western Blot—After treating with the designated compounds for the indicated time periods, cells were washed twice with ice-cold PBS. Cells lysis was done by adding radioimmunoprecipitation assay buffer with a protease inhibitor mixture. The protein concentration in the lysate was determined by BCA assay, and 30 μg of protein samples were mixed in 1:1 ratio with 2× Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, followed by heating at 95 °C for 5 min. The samples were run on 10% SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose membrane for Western blot analysis. The primary antibodies were used at 1:1000 dilution except for EAAT1 (1:5000 dilution), and HRP-conjugated secondary antibodies (Promega) were used at 1:5000 dilution. The blots were detected by an enhanced chemiluminescence detection kit (Pierce).

Cellular Fractionation—To prepare the cytoplasmic and nuclear extracts, the cells were first lysed in hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5% Nonidet P-40. The cell lysates were carefully collected with cell scraper and centrifuged at 2,500 rpm for 5 min at 4 °C. Thus, the obtained supernatant was cytoplasmic extract, and the pellet containing nuclei was incubated in hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol) on ice for 30 min with periodic vortexing. After spinning at 20,000 × *g* for 10 min at 4 °C, the nucleic extracts were collected and stored at −80 °C till use.

shRNA Lentiviral Particles Transduction—Cells were grown for 24 h in complete growth medium and YY1 or control shRNA lentiviral particles (20 multiplicity of infection) were added in medium containing 5 μg/ml of Polybrene. After 24 h of incubation, the cells were switched back to normal growth medium and further incubated overnight. The knockdown of YY1 was confirmed by mRNA and protein analysis. In some cases, the viral transduced cells were grown in the presence of puromycin dihydrochloride (8 μg/ml), and stable clones expressing YY1 shRNA were screened and used for further experiments.

Quantitative Real Time PCR (qPCR)—TRIzol reagent was used to extract total RNA from the cells, and 2 μg of RNA was transcribed to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies). Then for quantitative real time PCR for EAAT1, YY1, and GAPDH, the CFX96 real time PCR detection system (Bio-Rad) was used. The reactions mixture was comprised of 1 μg of cDNA template, 0.4 μM of the appropriate primers, and RT2 SYBR Green qPCR Master Mix (SA Biosciences/Qiagen) in a total volume of 25 μl. The PCR was programmed as 1 cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. A web-based PCR array data analysis program (SA Biosciences/Qiagen) was performed to analyze the data using GAPDH as an internal control. The primers used for PCR were as follows: for EAAT1, 5′-ACG GTC ACT GCT GTC ATT G-3′ (forward) and 5′-TGT GAC GAG ACT GGA GAT GA-3′ (reverse); for YY1, 5′-CTC CTG CAG CCC TGG GCG CAT C-3′ (forward) and 5′-GGT AAG CCC TTT AGC GCC TC-3′ (reverse); and for GAPDH, 5′-TCC CTC AAG ATT GTC AGC AA-3′ (forward) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (reverse).

Glutamate Uptake Assay—The glutamate uptake activity via EAAT1 was determined in the presence of dihydrokainic acid (DHK), a specific inhibitor for GLT-1, as described previously (51). Briefly, at the end point of all treatments, the cells were washed twice with prewarmed glutamate uptake buffer (122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, and 10 mM D-(+)-glucose, pH 7.4). Five μl of prewarmed uptake buffer containing 0.25 μCi/ml L-[^{3}H]glutamate (specific activity, 49.0 Ci/mmol; PerkinElmer Life Sciences) and 100 nM unlabeled glutamate was added to each well. After incubation for 10 min at 37 °C, the reaction was terminated by washing three times with ice-cold PBS, immediately followed by cell lysis in 1 ml of 1 N NaOH. An aliquot of 750 μl of each sample was transferred into scintillation vials and neutralized with 75 μl of 10 N HCl. Subsequently, 5 ml of liquid scintillation fluid was added to each vial, and the radioactivity was measured in liquid scintillation counter (LS 6500; Beckman Coulter). The uptake activity was calculated as nmol glutamate/mg protein/min after correcting with protein concentrations as determined by BCA assay.

Co-immunoprecipitation (Co-IP)—For Co-IP experiments, 400 μg of nuclear extracts were incubated with 2 μg of the indicated antibodies and rocked at 4 °C for an hour. Then 20 μl

of protein A+G-agarose beads (Santa Cruz) were added and incubated overnight. After washing the beads three times with radioimmunoprecipitation assay buffer, 40 μl of 2× SDS sample buffer was added and boiled at 95 °C for 3 min. After spinning, the eluted proteins were obtained on the supernatant, which was used for running for SDS-PAGE, followed by Western blot.

Immunocytochemistry—Cells grown on coverslips were fixed in methanol (−20 °C), followed by blocking (5% goat serum in PBS with 0.1% Triton X-100) for 1 h. Cells were then incubated with primary antibodies specific to HDAC2 (1:100, Santa Cruz), YY1 (1:100, Santa Cruz) or p65 (1:100, Santa Cruz) overnight at 4 °C. After washing three times (PBS with 0.1% Triton X-100), anti-goat (FITC-conjugated), anti-rabbit (rhodamine-conjugated), or anti-mouse (FITC-conjugated) secondary antibodies (1:100, Abcam) were added and incubated for 1 h at room temperature. Cells were also counterstained with DAPI (50 μg/ml stock in PBS, 1:50 dilution; Sigma-Aldrich) and mounted on slides with ProLong® Gold antifade mountant (Molecular Probes). The images were taken with an A1R laser scanning confocal microscope (Nikon) with NIS-Elements software.

Chromatin Immunoprecipitation Assay (ChIP)—EZ-ChIP kit from Millipore was used to perform a ChIP assay, following the manufacturer's instructions. Briefly, at the end of the treatment, cells were treated with formaldehyde for 10 min at room temperature to obtain the cross-linking of protein-DNA complexes. Then sonication was performed to get the chromatins of lengths of 100–500 bp. After centrifugation, 900 μl of ChIP dilution buffer was added to 100 μl of supernatant. After preclearing with agarose beads, 1% of the reaction was saved as input, and the rest was incubated overnight at 4 °C with p65, p50, YY1, or rabbit IgG (negative control) antibodies. Following the isolation and washing of antibody-containing complexes, DNA was purified to run PCR with the following primers: for EAAT1, 5′-GCG TGA AAG TGG TCT AAG GAG-3′ (forward) and 5′-GCA AGT TAC TAT CAG GGC AAC-3′ (reverse); and for YY1, 5′-CTT TGC AAC ACA GGC AGT GTG-3′ (forward) and 5′-GTG TGC TCT TTA GCC TGG TTG-3′ (reverse). PCR products were resolved on 1% agarose gel and visualized under UV light.

Electrophoretic Mobility Shift Assay (EMSA)—The HPLC-purified and biotin-labeled oligonucleotides containing the consensus sites for NF-κB or YY1 on the EAAT1 promoter were purchased from Eurofins MWG Operon, and EMSA was carried out with a LightShift chemiluminescent kit from Pierce. Briefly, 5 μg of nuclear extract from control or treated cells was incubated for 20 min on ice with biotin-labeled oligonucleotides. Then DNA-protein complexes were resolved in 6% DNA retardation gels (Life Technologies) and transferred to nylon membrane. The complexes were detected with the chemiluminescent nucleic acid detection module from Pierce. The primers pairs used were as follows: for EAAT1 −116 NF-κB, 5′-CAG AAA CCT CGG GGT TTC CCC CTC CTC CCT G-3′ (forward) and 5′-CAG GGA GGA GGG GGA AAC CCC GAG GTT TCT G-3′ (reverse); for EAAT1 −538 NF-κB, 5′-GAA ATA GAG GCA TGT CCC TAA CTT TAG AC-3′ (forward) and 5′-GTC TAA AGT TAG GGA CAT GCC TCT ATT TC-3′ (reverse); and for EAAT1 YY1 (−482), 5′-TTT TTT TTC TAC ATA CTT GTC TGC ATT CAG-3′ (forward) and 5′-CTG AAT GCA GAC AAG TAT GTA GAA AAA AAA-3′ (reverse).

DNA Affinity Purification Assay (DAPA)—The FactorFinder Kit from Militenyi Biotech Inc. (Auburn, CA) was used to run DAPA with biotinylated oligonucleotides. Briefly, 1.5 μg of biotinylated oligonucleotides containing the consensus sites for NF-κB or YY1 on the EAAT1 promoter was incubated with 50 μg of nuclear extract in binding buffer for 20 min. After adding 100 μl of streptavidin microbeads, the reaction mixture was further incubated for 10 min. Then the mixture was applied onto the μ column, which was pre-equilibrated with binding buffer and set in the magnetic field. After 4 × 100 μl of each washing with low salt and high salt buffers, specifically bound proteins were eluted with 30 μl of elution buffer provided in the kit and analyzed by Western blotting.

Statistical Analysis—The mean ± S.E. was calculated from the data in each group and statistical analysis was performed using GraphPad Prism software (GraphPad Inc., La Jolla, CA). Statistical differences between control and treated groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test with statistical significance set at $p < 0.05$. Each experiment was carried out in at least three independently prepared astrocytes. The number of samples for each experiment was at least three for Western blot and real time PCR analyses and six for glutamate uptake and luciferase assays.

Results

NF-κB Is a Positive Regulator of EAAT1 and Mediates EGF-induced Increase in EAAT1 Expression—The promoter regions of both EAAT1 (human form) and GLAST (rodent form) contain consensus sequences of NF-κB and YY1 (Fig. 1*A*) (the rat promoter is not shown). We found nine putative YY1 binding sites and two NF-κB sites in the EAAT1 promoter region (from −1 to −1973). NF-κB plays a critical role in enhancing EAAT1 (or GLAST in rodents) expression induced by EGF and raloxifene in both rat and human astrocytes (19, 21), whereas YY1 plays an important role in reduction of EAAT1 mRNA levels in chick astrocytes (30). EAAT1 promoter activity is increased by EGF (18–20) but decreased by manganese (21, 22) in rat and human astrocytes. This indicates that both NF-κB and YY1 play critical roles in EAAT1 regulation in rodents and human astrocytes. In the present study, we used rat primary astrocytes and human astrocytic H4 cells, which were used to study the regulation of EAAT1 (18) and EAAT2 (24). We first confirmed that H4 cells respond to EGF and manganese in a similar way to rat primary astrocytes (data not shown). Because several studies, including ours (19, 21), indicate that NF-κB plays a critical role in EAAT1 regulation, we explored the molecular mechanisms of NF-κB-induced positive regulation of EAAT1 at the transcriptional level. We identified two NF-κB binding sites (−116 and −538) and multiple YY1 binding sites (nine sites) in EAAT1 promoter sequences (Fig. 1*A*).

The results showed that overexpression of NF-κB p65 robustly increased EAAT1 promoter activity (Fig. 1*B*), as well as EAAT1 mRNA and protein levels (Fig. 1, *C* and *D*) in rat astrocytes or human astrocyte H4 cells. Both cell types showed analogous effects, and therefore we used these two cell types interchangeably. Overexpression of IκBα, an inhibitor of NF-κB

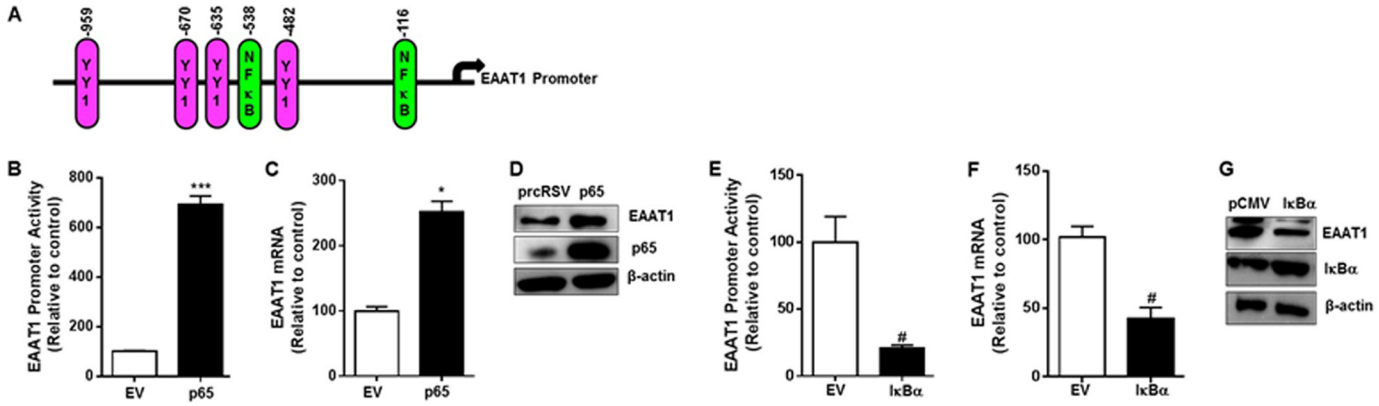

FIGURE 1. **NF-κB positively regulates EAAT1.** *A*, putative consensus sites for NF-κB and YY1 in the EAAT1 promoter; for simplicity, *cis*-elements of these transcription factors located up to −1000 bp in the EAAT1 promoter are shown. *B*, human H4 astrocytes were co-transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid and 0.1 μg of either control empty vector prcRSV (*EV*) or NF-κB p65, followed by luciferase assay to determine EAAT1 promoter activity as described under "Experimental Procedures." *C*, cells were transfected overnight with 2 μg of either control EV or NF-κB p65, and EAAT1 mRNA levels were determined by qPCR. *D*, after overnight transfection with either control EV or NF-κB p65, cell lysates were prepared, and Western blotting was carried out to determine EAAT1 protein levels. The same blots were reprobed for p65 and β-actin. *E*, cells were co-transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid and 0.1 μg of either empty vector pCMV (EV) or IκBα, followed by luciferase assay. *F* and *G*, cells were transfected overnight with 2 μg of either EV or IκBα plasmids, followed by EAAT1 mRNA (*F*) and protein analysis (*G*) (***, $p < 0.001$; *, $p < 0.05$; #, $p < 0.05$; ANOVA followed by Tukey's post hoc test; $n = 3–4$).

nuclear translocation by sequestering it in the cytosol, decreased EAAT1 promoter activity, mRNA, and protein levels (Fig. 1, *E–G*). Pharmacological inhibition of NF-κB by pyrrolidine dithiocarbamate and QNZ also decreased EAAT1 promoter activity (Fig. 2*A*) and abrogated EGF-induced enhancement of EAAT1 promoter activity, as well as EAAT1 mRNA levels and EAAT1-mediated glutamate uptake (EAAT2-mediated glutamate uptake was blocked with 100 μM of DHK, a specific inhibitor of EAAT2) (Fig. 2, *A–D*). These results indicate that the NF-κB pathway plays a critical role in mediating the stimulatory effects of EGF on EAAT1 expression and glutamate uptake.

NF-κB Consensus Sites (−116 and −538) in the EAAT1 Promoter Are Critical for Positive Regulation—The EAAT1 promoter revealed two putative sites for NF-κB *cis*-elements at −116 and −538 position (Fig. 1*A*). To determine whether they are critical sites for NF-κB effect on EAAT1 regulation, the sites were mutated, and EAAT1 promoter activity was determined. The results showed that mutation of either of these two NF-κB binding sites significantly decreased EAAT1 promoter activity ($p < 0.001$; Fig. 3*A*). Double mutations further decreased the EAAT1 promoter activity significantly compared with single mutations ($p < 0.05$ with −116, and $p < 0.001$ with −538 mutants), suggesting that both NF-κB sites are critical for the positive regulation of EAAT1. There was also a significant reduction in NF-κB p65 overexpression-induced increase in EAAT1 promoter activity in NF-κB single mutants of EAAT1 (~7-fold increase in wild type *versus* ~3-fold increase in both single mutants; Fig. 3*B*). More importantly, NF-κB overexpression did not increase the EAAT1 promoter activity in the double mutant, suggesting that both NF-κB binding sites are essential for mediating the stimulatory effects of NF-κB on EAAT1. We also attempted to determine the significance of these NF-κB binding sites for EGF-induced stimulatory effects on EAAT1. EGF still increased EAAT1 promoter activity in either of the NF-κB binding site mutants, but double mutations completely abolished the EGF effect on EAAT1 promoter activity (Fig. 3*C*), further indicating that both NF-κB sites are critical for positive regulation of EGF on EAAT1.

EGF Induces Nuclear Translocation of NF-κB p65 and Binding to the EAAT1 Promoter Regions—Because activation of NF-κB requires its nuclear translocation from the cytosol, where it is sequestered by IκB, we investigated whether EGF activates NF-κB via this classical mechanism. Activation of NF-κB is facilitated by its phosphorylation and IκB degradation prior to nuclear translocation of NF-κB (52, 53). EGF-treated cells were fractionated into cytoplasmic and nuclear extracts, and protein levels of IκBα and NF-κB were determined. The results showed that NF-κB p65 protein levels in the nuclear fraction were significantly increased as early as 15 min after EGF treatment, with a parallel reduction of IκBα in cytoplasmic extracts (Fig. 3*D*).

We also determined whether EGF induces NF-κB binding to its consensus sites in the EAAT1 promoter by ChIP, EMSA, and DAPA assays. The *in vivo* binding of NF-κB to the EAAT1 promoter was analyzed by the ChIP assay. The results showed that EGF significantly increased binding of both NF-κB subunits p65 and p50 to the EAAT1 promoter (Fig. 4*A*). DAPA and EMSA assays were also performed to determine *in vitro* binding of NF-κB to their consensus sites in EAAT1 promoter in the nuclear extract of EGF-treated astrocytes. Results by both DAPA (Fig. 4*B*) and EMSA (Fig. 4*C*) showed that EGF induced NF-κB binding to both consensus sites (−116 and −538) in the EAAT1 promoter. This was supported by the results that the specific binding of biotinylated NF-κB oligonucleotides to the EAAT1 promoter was blocked by the addition of excess amounts of nonbiotinylated control oligonucleotides.

YY1 Is a Negative Regulator of EAAT1—A few studies suggest the role of YY1 in the inhibition of EAAT1 expression and function (29, 30). YY1 is a critical repressor in the regulation of EAAT2 (34, 54). Thus, we explored the role of YY1 in the regulation of EAAT1 at the transcriptional level. First, we tested whether overexpression of YY1 modulates EAAT1 expression in H4 cells. The results showed that YY1 decreased EAAT1

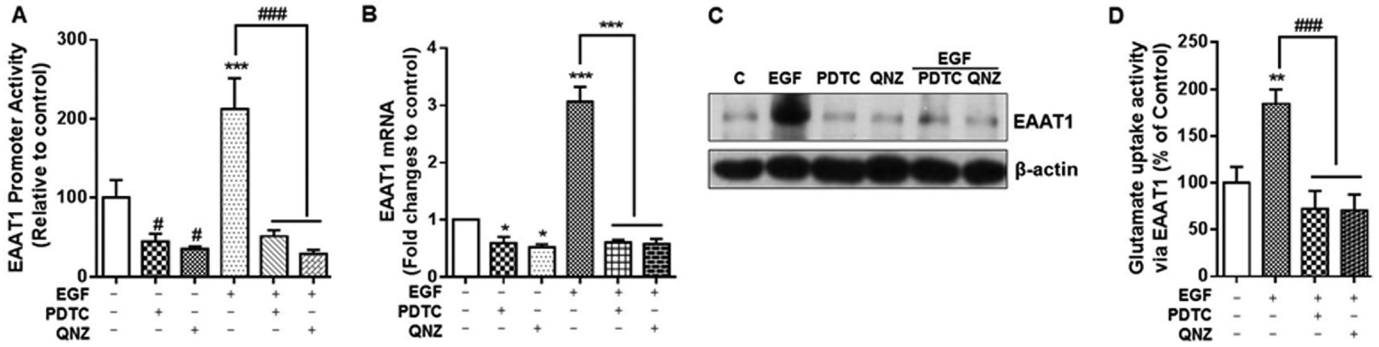

FIGURE 2. **NF-κB mediates EGF-induced EAAT1 expression and function.** *A*, human H4 astrocytes were transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid and then pretreated with 50 μM of PDTC or QNZ for 30 min, followed by treatment with 50 ng/ml of EGF for 24 h. EAAT1 promoter activity was determined by luciferase assay. *B* and *C*, cells were pretreated with 50 μM of PDTC or QNZ for 30 min, followed by treatment with 50 ng/ml of EGF for 24 h. EAAT1 mRNA levels were determined by qPCR (*B*), and protein levels were determined by Western blotting (*C*). *D*, after treatment with PDTC, QNZ, and EGF, astrocytes were tested for glutamate uptake in the presence of 100 μM of DHK to inhibit GLT-1 as described under "Experimental Procedures" (**, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$; ###, $p < 0.001$; ANOVA followed by Tukey's post hoc test; $n = 3–4$).

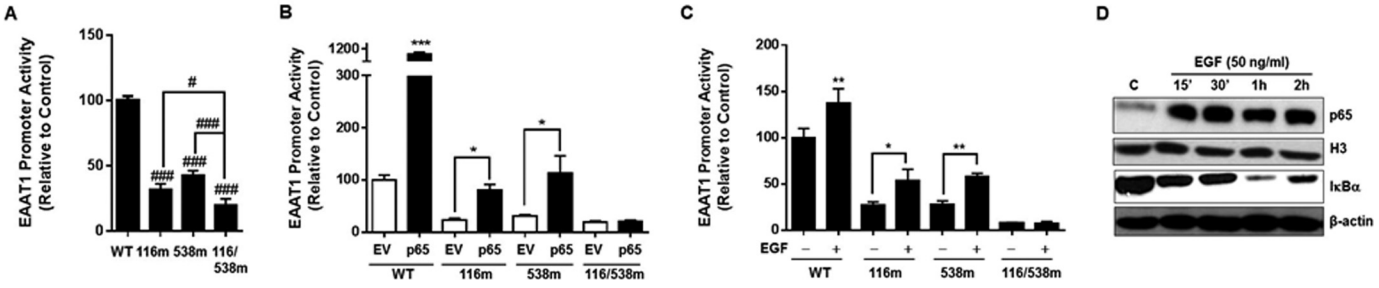

FIGURE 3. **NF-κB sites are critical for positive regulation of EAAT1 and EGF induces nuclear translocation of NF-κB.** *A*, NF-κB binding sites of EAAT1 promoter (−116 and −538 position) were mutated by site-directed mutagenesis, and human H4 astrocytes were transfected overnight with 0.5 μg of WT, −116 mutant (*116m*), −538 mutant (*538m*), or both −116 and −538 mutants (*116/538m*), followed by luciferase assay to determine EAAT1 promoter activity. *B*, cells were co-transfected overnight with 0.5 μg of WT or NF-κB mutant EAAT1 promoter luciferase plasmids and 0.1 μg of either empty vector prcRSV (*EV*) or NF-κB p65, followed by luciferase assay. *C*, after being transfected overnight with 0.5 μg of WT or NF-κB mutants of EAAT1 promoter, astrocytes were treated with 50 ng/ml of EGF for 24 h, followed by luciferase assay. *D*, cells were treated with 50 ng/ml of EGF for the indicated time periods, followed by the preparation of cytoplasmic and nuclear extracts. Western blot was carried out with equal amounts of cytoplasmic and nuclear fractions to detect IκBα and NF-κB p65, respectively. Histone H3 and β-actin were used as loading controls for nuclear and cytoplasmic extracts, respectively (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$; ###, $p < 0.001$; ANOVA followed by Tukey's post hoc test; $n = 3$).

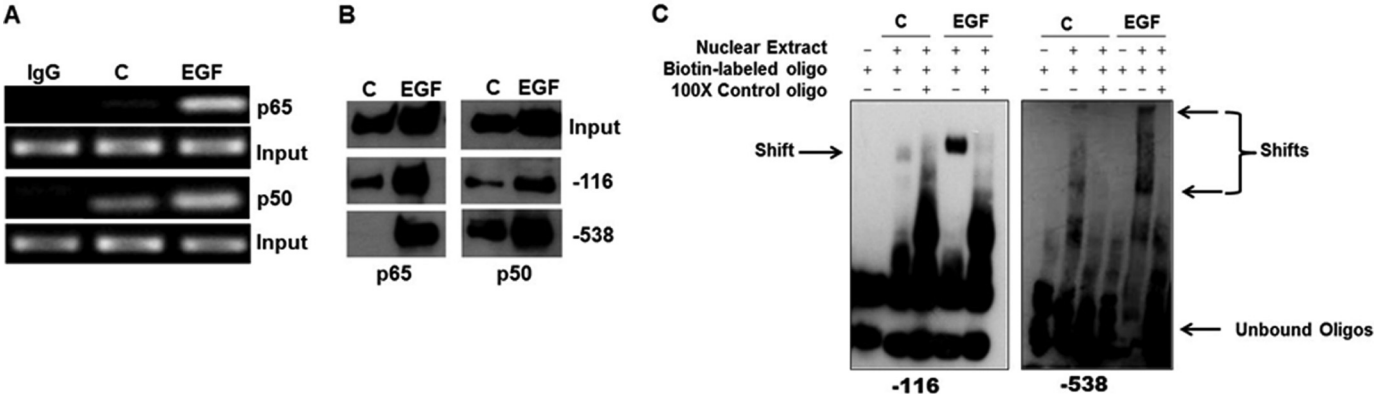

FIGURE 4. **EGF induces NF-κB binding to the EAAT1 promoter.** *A*, human H4 astrocytes were treated with EGF (50 ng/ml, 2 h), followed by ChIP assay to detect bindings of NF-κB p50 and p65 isoforms to the EAAT1 promoter as described under "Experimental Procedures." Rabbit IgG was used as a negative control for vehicle-control group and EGF-treated group. *B*, nuclear extracts were prepared from EGF-treated astrocytes, and DAPA was performed with biotinylated oligonucleotides for both NF-κB binding sites (−116 and −538). The eluted proteins were run for Western blot to detect p50 and p65. Input controls for both p65 and p50 are also presented. *C*, EMSA was performed with nuclear extracts for −116 (*left panel*) and −538 (*right panel*) NF-κB binding sites of the EAAT1 promoter. The shifts containing the DNA-protein complex are shown with *arrows*. *Lanes C*, control.

promoter activity (Fig. 5*A*), as well as EAAT1 mRNA and protein levels (Fig. 5, *B* and *C*). To further validate the negative role of YY1 in EAAT1 expression, shRNA lentiviral particles were transduced to knock down YY1. The results showed that reduced expression of YY1 increased EAAT1 protein expression (Fig. 5*D*), indicating that YY1 is a repressor of EAAT1.

YY1 Mediates Manganese-induced Repression of EAAT1 Expression and Function—It is well established that manganese inhibits expression and function of astrocytic glutamate transporters including EAAT1 (22, 55), but the molecular mechanisms involved at the transcriptional level are not completely understood. YY1 is a critical transcriptional mediator of man-

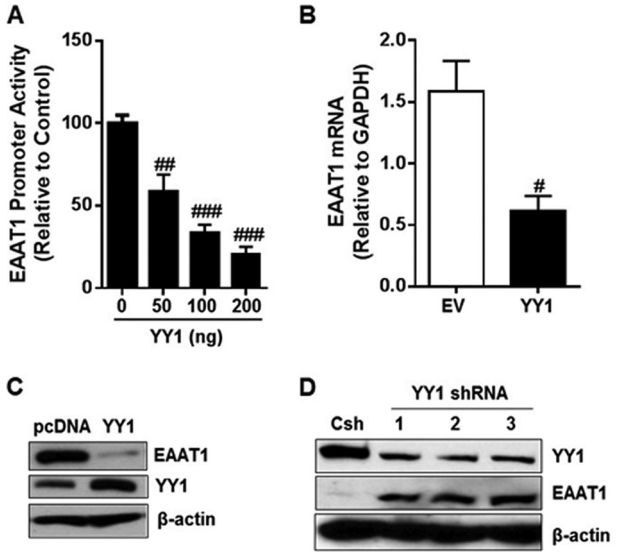

FIGURE 5. **YY1 negatively regulates EAAT1.** *A*, human H4 astrocytes were co-transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid and 0.1 μg of either empty vector pcDNA (*EV*) or the indicated concentrations of YY1, followed by luciferase assay to determine EAAT1 promoter activity. *B*, cells were transfected overnight with 2 μg of either EV or YY1, and EAAT1 mRNA levels were determined by qPCR. *C*, after overnight transfection with either EV or YY1, EAAT1 protein levels were determined by Western blotting. The same blots were reprobed for YY1 to confirm the overexpression of YY1 and also for β-actin to ensure equal loading. *D*, cells were infected with control shRNA (*Csh*) or YY1 shRNA lentiviral particles for 24 h, and cells stably expressing YY1 shRNA were screened with puromycin selection as described under "Experimental Procedures." Cell lysates were prepared from control and three different clones (*lanes 1–3*) of YY1 shRNA expressing cells and run for Western blotting to detect YY1. The same blot was reprobed for EAAT1 and β-actin (#, $p < 0.05$; ###, $p < 0.001$; ANOVA followed by Tukey's post hoc test; $n = 3$).

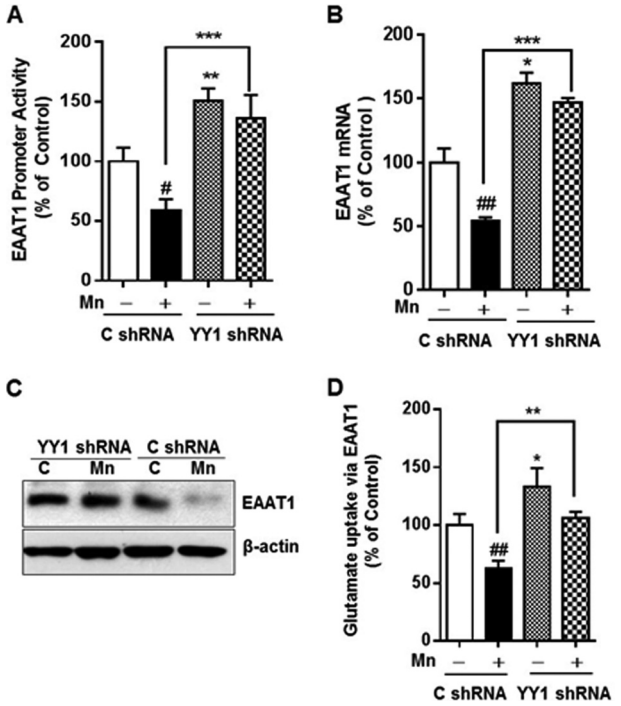

FIGURE 6. **YY1 knockdown reverses manganese-induced repression of EAAT1 expression and function.** *A*, human H4 astrocytes stably transduced with control or YY1 shRNA were transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid, followed by treatment with 250 μM of manganese for 6 h. Then EAAT1 promoter activity was determined by luciferase assay. *B* and *C*, cells stably expressing control shRNA (*C shRNA*) or YY1 shRNA were treated with 250 μM of manganese for 6 h, followed by the determination of EAAT1 mRNA and protein levels by qPCR and Western blotting, respectively. *D*, cells stably expressing control or YY1 shRNA were treated with 250 μM of manganese for 6 h, and glutamate uptake via EAAT1 was determined after pretreating cells with 100 μM of DHK for 30 min (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$; ##, $p < 0.01$; ANOVA followed by Tukey's post hoc test; $n = 3$–4).

ganese-induced inhibition of EAAT2 (34). To determine whether YY1 plays a role in manganese-induced repression of EAAT1 at the transcriptional level, we first measured manganese-induced modulation of EAAT1 promoter activity in YY1 knockdown conditions with YY1 shRNA lentiviral particles. The results showed that YY1 knockdown reversed manganese-induced repression of EAAT1 promoter activity ($p < 0.001$; Fig. 6*A*), as well as EAAT1 mRNA ($p < 0.001$; Fig. 6*B*) and protein levels (Fig. 6*C*). YY1 knockdown also reversed manganese-induced decrease in glutamate uptake activity via EAAT1 ($p < 0.01$; Fig. 6*D*).

Manganese Induces YY1 Recruitment to Its Binding Sites in the EAAT1 Promoter in Vivo and in Vitro—Manganese increases YY1 promoter activity and mRNA/protein levels in astrocytes (34). Thus, we tested whether manganese induces YY1 binding to its consensus sites in the EAAT1 promoter both *in vivo* and *in vitro*. The results showed that manganese increased YY1 binding to its consensus site in the EAAT1 promoter *in vivo* as shown by the ChIP assay (Fig. 7*A*), as well as *in vitro* as shown by EMSA and DAPA (Fig. 7, *B* and *C*).

YY1 Interacts with NF-κB p65 and Overrides p65 Stimulatory Effects on EAAT1 Promoter Activity—Because manganese activates NF-κB (56), but NF-κB is a main positive regulator of EAAT1, we addressed the mechanism by which manganese represses EAAT1. We found that p65 robustly increased, whereas YY1 decreased, EAAT1 promoter activity (Fig. 8*A*), but co-expression of both NF-κB p65 and YY1 completely abol-

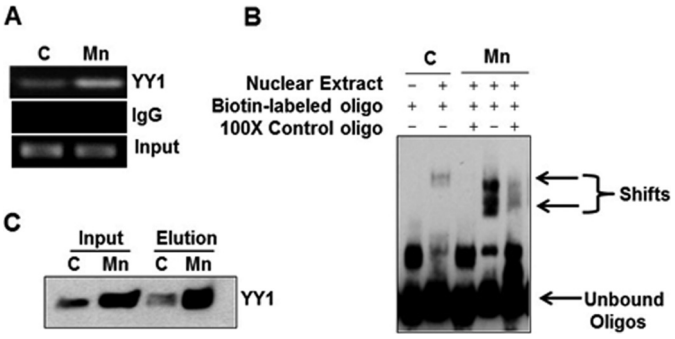

FIGURE 7. **Manganese increases YY1 binding to the EAAT1 promoter.** *A*, human H4 astrocytes were treated with manganese (250 μM, 6 h), followed by ChIP assay with YY1 as described under "Experimental Procedures." Rabbit IgG was used as a negative control, and inputs for all the samples are also shown. *B*, nuclear extracts prepared from manganese-treated (250 μM, 6 h) cells were used to perform EMSA with the biotinylated oligonucleotides containing YY1 binding sites (−482 position) of the EAAT1 promoter. The shifts containing the DNA-protein complex are shown with *arrows*. *C*, DAPA was performed for the same YY1 binding site containing biotinylated oligonucleotides and nuclear extracts, followed by Western blotting for YY1. *Lanes C*, control.

ished the stimulatory effects of NF-κB p65, suggesting that YY1 is dominant over NF-κB p65. The NF-κB pathway is activated by positive modulators of EAAT1 like EGF, but some negative modulators like TNF-α are also known to activate NF-κB (24). Moreover, manganese increased the physical interaction

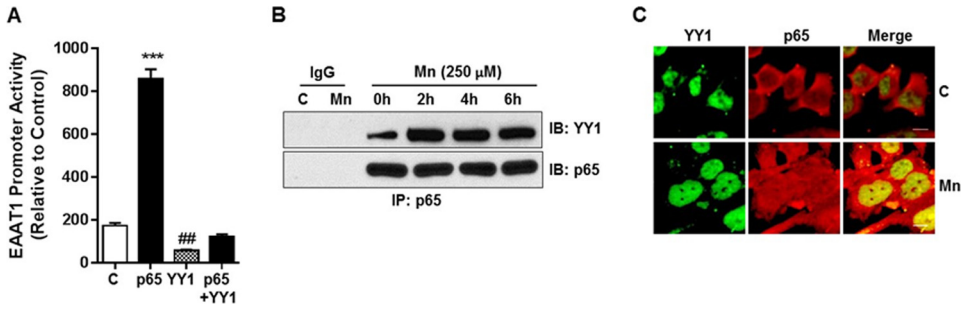

FIGURE 8. **YY1 interacts with p65 and overrides the stimulatory effects of p65 on EAAT1 promoter activity.** *A*, human H4 astrocytes were transfected overnight with YY1 or p65 alone or co-transfected with both YY1 and p65, followed by luciferase assay to determine EAAT1 promoter activity. *B*, cells were treated with manganese (250 μM) for the indicated period of time, and nuclear extracts were used to run Co-IP for YY1 and p65. Rabbit IgG was used as a negative control for vehicle-control and manganese-treated nuclear extracts. *C*, after rat primary astrocytes were treated with manganese (250 μM, 6 h), immunocytochemistry was performed to detect YY1 and p65 using a confocal microscope (Nikon, A1R). Representative images are shown (***, $p < 0.001$; ##, $p < 0.01$; ANOVA followed by Tukey's post hoc test; $n = 3$). *Scale bar*, 10 μm. *Row C*, control; *IB*, immunoblot; *IP*, immunoprecipitation.

between YY1 and p65 as evidenced by Co-IP (Fig. 8*B*). Further, confocal imaging also showed the increased co-localization of these two proteins in the nucleus after manganese treatment (Fig. 8*C*), indicating that manganese-enhanced interaction between the two proteins occurred in the nucleus. It suggests that manganese-induced activation of YY1 overrides NF-κB p65 by physical sequestration when both pathways are activated, ultimately leading to repression of EAAT1.

YY1 Recruits HDACs as Co-repressors of EAAT1 Promoter Activity—Because YY1 often recruits epigenetic modifiers such as HDACs to repress gene expression (57), we tested whether the latter are recruited during YY1-induced repression of EAAT1 promoter activity. We tested HDAC1 and HDAC2 as class I HDACs and HDAC4 and HDAC5 as class II HDACs. The results revealed that overexpression of either HDAC1 or HDAC2 significantly decreased EAAT1 promoter activity ($p < 0.001$), and co-expression of either HDAC1 or HDAC2 with YY1 further decreased EAAT1 promoter activity, compared with YY1 alone ($p < 0.05$; Fig. 9*A*). Similarly, overexpression of either HDAC4 or HDAC5 also decreased EAAT1 promoter activity ($p < 0.001$), and co-expression with YY1 further decreased EAAT1 promoter activity (Fig. 9*B*). Physical interaction of either HDAC2 or HDAC4 with YY1 was confirmed by Co-IP, and these interactions were increased by manganese (Fig. 9, *C* and *D*). We also employed immunocytochemistry to study the subcellular localization of interaction between HDAC2 and YY1. The confocal images revealed the increased co-localization between HDAC2 and YY1 in the nucleus after manganese treatment (Fig. 9*E*), indicating that manganese increased the interaction of YY1 and HDAC2 mostly in the nucleus. These results indicate that HDACs are epigenetic co-repressors of YY1 and inhibit EAAT1 promoter activity.

HDACs Inhibit p65 Stimulatory Effects on EAAT1 Promoter Activity—To further understand the mechanism of EAAT1 repression at the transcriptional level via HDACs, we also tested whether HDACs interact with NF-κB. The results showed that co-expression of either HDAC1 or HDAC2 from class I with p65 completely abolished the NF-κB p65 stimulatory effects on EAAT1 promoter activity (Fig. 10*A*). Class II HDACs, HDAC4 and HDAC5, also completely inhibited the p65-induced increase in EAAT1 promoter activity when they were co-expressed (Fig. 10*B*). Manganese increased physical interactions between HDAC2 and p65 (Fig. 10*C*), as well as between HDAC4 and p65 (Fig. 10*D*). The increased interaction between HDAC2 and p65 following manganese treatment was localized in the nucleus, as shown by confocal imaging (Fig. 10*E*). These results suggest that HDACs, YY1 and NF-κB form a large complex to repress EAAT1 promoter activity, which is enhanced by manganese.

Inhibition of HDACs Increases EAAT1 Expression and Reverses Manganese-induced Repression of EAAT1—Because HDAC inhibitor TSA has been shown to increase EAAT1 transcription (46) and overexpression of HDACs repressed EAAT1 in the present study, we examined various inhibitors of HDAC class I and II to see whether they would increase EAAT1 expression and attenuate manganese-induced inhibition of EAAT1. Our results showed that all HDAC inhibitors tested, including FK228, TSA, VPA, NaB, and suberoylanilide hydroxamic acid, increased EAAT1 promoter activity, as well as EAAT1 mRNA/protein levels. These effects were more pronounced with VPA, suberoylanilide hydroxamic acid, and NaB compared with FK228 and TSA (Fig. 11, *A–C*). These HDAC inhibitors also reversed manganese-induced down-regulation of EAAT1. More importantly, these HDAC inhibitors increased glutamate uptake via EAAT1 and also reversed the manganese-induced reduction in glutamate uptake (Fig. 11*D*). These results suggest that HDAC inhibitors enhance both the expression and function of EAAT1, as well as reverse manganese-induced reduction in EAAT1 expression and function.

Discussion

EAAT1 (GLAST in rodents) is one of two main glutamate transporters that removes excess glutamate from the synaptic clefts, thus preventing excitotoxic neuronal death. Yet the mechanism of the transcriptional regulation of EAAT1 has to be established. Our findings demonstrate that NF-κB is a major positive regulator of EAAT1, mediating the stimulatory effects of the positive modulator EGF on EAAT1 expression by the canonical activation of the NF-κB pathway and induction of NF-κB binding to the EAAT1 promoter (Figs. 1–4). The results also reveal that YY1 is a critical repressor of EAAT1, mediating the inhibitory effects of manganese on EAAT1 expression (Figs. 5 and 6). Moreover, YY1 recruits HDACs as co-repressors to

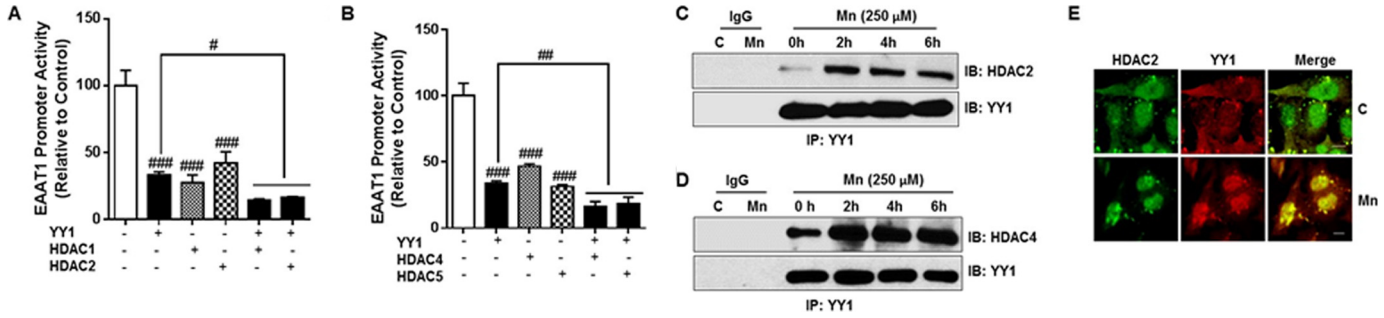

FIGURE 9. **YY1 recruits HDACs as co-repressors to inhibit EAAT1.** *A* and *B*, human H4 astrocytes were transfected overnight with YY1 or the designated isoforms of HDACs alone or co-transfected with both YY1 and HDACs, followed by measuring EAAT1 promoter activity. *C* and *D*, cells were treated with manganese (250 μM) for the indicated time periods, and nuclear extracts were prepared for Co-IP to determine interaction between YY1 and HDAC2 (*C*) or HDAC4 (*D*). Rabbit IgG was used as a negative control for vehicle-control and manganese-treated nuclear extracts. *E*, after rat primary astrocytes were treated with manganese (250 μM, 6 h), immunocytochemistry was performed to detect YY1 and HDAC2 using a confocal microscope (Nikon, A1R). Representative images are shown (#, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ANOVA followed by Tukey's post hoc test; $n = 3$). *Scale bar*, 10 μm. *Row C*, control; *IB*, immunoblot; *IP*, immunoprecipitation.

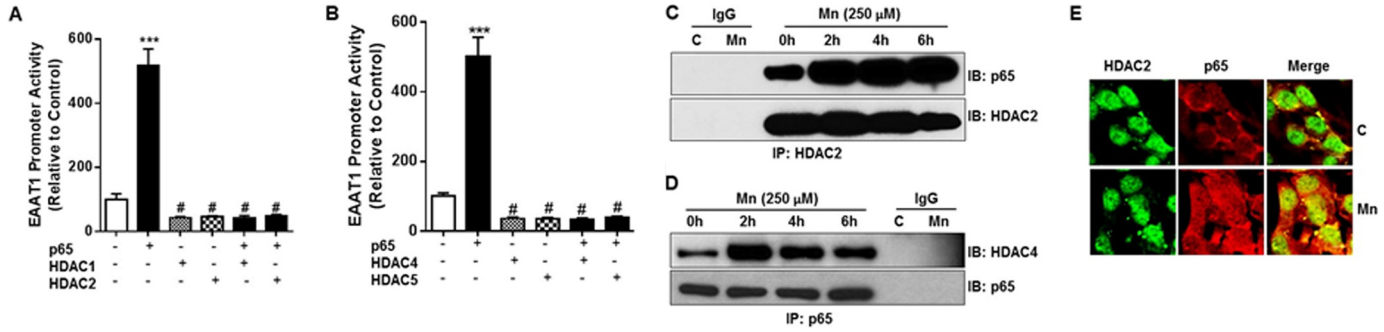

FIGURE 10. **HDACs interact with p65 and override the stimulatory effects of p65 on EAAT1.** *A* and *B*, human H4 astrocytes were transfected overnight with either p65 or designated HDAC isoforms, as well as co-transfected with both p65 and HDACs, followed by luciferase assay to determine EAAT1 promoter activity. *C* and *D*, cells were treated with manganese (250 μM) for the indicated time periods, and nuclear extracts were prepared for Co-IP for p65 and HDAC2 (*C*) or for p65 and HDAC4 (*D*). Rabbit IgG was used as a negative control for both vehicle-control and manganese-treated nuclear extracts. *E*, after rat primary astrocytes were treated with manganese (250 μM, 6 h), immunocytochemistry was performed to detect p65 and HDAC2 using a confocal microscope (Nikon, A1R). Representative images are shown (***, $p < 0.001$; #, $p < 0.05$; ANOVA followed by Tukey's post hoc test; $n = 3$). *Scale bar*, 10 μm. *Row C*, control; *IB*, immunoblot; *IP*, immunoprecipitation.

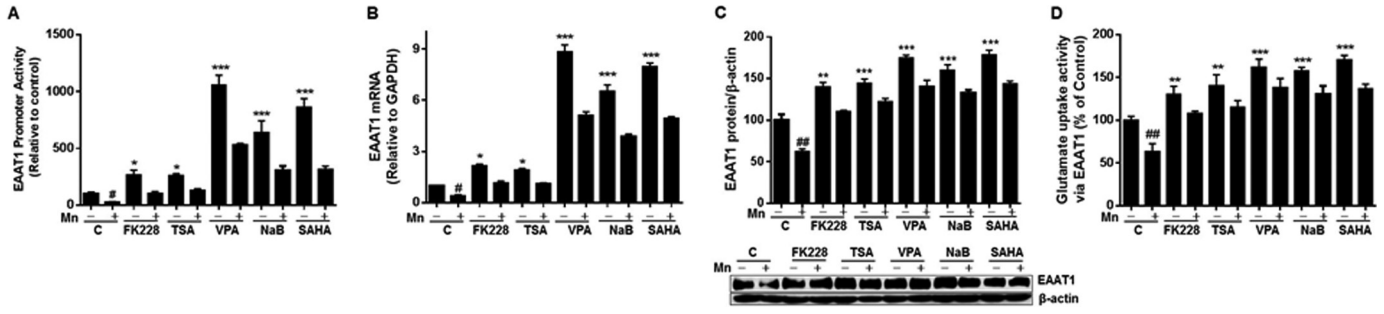

FIGURE 11. **HDAC inhibitors reverse manganese-induced repression of EAAT1 expression.** *A*, after transfected overnight with 0.5 μg of EAAT1 promoter luciferase plasmid, human H4 astrocytes were treated with FK228 (10 nM), TSA (200 μM), VPA (1 mM), NaB (1 mM), or suberoylanilide hydroxamic acid (1 μM) for 24 h, followed by manganese (250 μM, 6 h) exposure and subsequent luciferase assay to determine EAAT1 promoter activity. *B* and *C*, astrocytes were treated with the same compounds and conditions described in *A*, followed by determination of EAAT1 mRNA (*B*) and protein (*C*) levels by qPCR and Western blotting, respectively. Quantification of band density for EAAT1 proteins is shown in *D* (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$; ##, $p < 0.01$; ANOVA followed by Tukey's post hoc test; $n = 3$). *D*, cells were treated with various HDAC inhibitors and manganese as described in *A*, and glutamate uptake via EAAT1 was determined after pretreating cells with 100 μM of DHK (an EAAT2 inhibitor) for 30 min (**, $p < 0.01$; ***, $p < 0.001$; ##, $p < 0.01$; ANOVA followed by Tukey's post hoc test; $n = 3$). *Lanes C*, control; *SAHA*, suberoylanilide hydroxamic acid.

further inhibit EAAT1 and overrides the stimulatory effects of NF-κB on EAAT1 promoter activity (Figs. 8 and 9), suggesting that manganese-induced enhancement of the YY1-HDACs-p65 complex plays a critical role in the repression of EAAT1 transcription.

It is well established that EAAT1 and EAAT2 are responsible for clearing excess glutamate from the synaptic clefts to prevent excitotoxic neuronal death (12, 13, 58). Although expressed at high levels in the cerebellum and retina (2, 3, 59), EAAT1 is expressed throughout the brain including cortical regions, particularly during development (60). Moreover, EAAT1 is expressed abundantly in cultured astrocytes, whereas EAAT2 is expressed at relatively low levels, and its expression depends on neuronal presence (61, 62), indicating different regulatory mechanisms for EAAT1 compared with EAAT2. Despite its critical involvement in physiological and pathological condi-

tions of the CNS (63), the transcriptional regulatory mechanisms of EAAT1 are largely unexplored.

The EAAT1 promoter sequences contain putative binding sites for various transcription factors including stimulating proteins 1 and 3 (Sp1 and Sp3) and cAMP-responsive element binding protein, demonstrating that Sp1 and 3 binding sites are critical for positive regulation of EAAT1 promoter activity (18). We also observed that Sp1 significantly increased EAAT1 promoter activity (2-fold, data not shown), but NF-κB exerts a greater potency (~7-fold increase) in activating the EAAT1 promoter and significantly increases EAAT1 mRNA/protein levels (Fig. 1), suggesting that NF-κB is the main positive regulator of EAAT1. This NF-κB-induced increase in EAAT1 promoter activity is also greater than its effect on EAAT2 promoter activity (7-fold *versus* 2-fold) (26). Although the positive effect of NF-κB on EAAT1 has been previously shown to mediate raloxifene-induced enhancement of EAAT1 expression (21), the present study further characterized the role of NF-κB at the promoter level, identifying that two NF-κB binding sites in the EAAT1 promoter, −116 and −538, are critical for the positive regulation of EAAT1 promoter activity (Fig. 3).

Several growth factors including EGF have been shown to increase EAAT1 expression, yet the mechanism by which EGF induces EAAT1 expression at the transcriptional level is not completely understood. One study has reported that stimulatory effects of EGF or TGF-α on EAAT1 protein expression were abolished by pharmacological inhibition of NF-κB with PDTC (19). Our finding that mutations in the NF-κB binding sites in the EAAT1 promoter abolished EGF-induced increase of EAAT1 promoter activity indicates that NF-κB activity via these two binding sites in the EAAT1 promoter are responsible for EGF-induced enhancement of EAAT1 promoter activity. EGF activates NF-κB via the canonical pathway to stimulate EAAT1 by increasing nuclear translocation of NF-κB, parallel with degradation of IκBα in the cytosol (Fig. 3*D*). These results are consistent with the previous report that EGF activates NF-κB via the degradation of IκBα (64). One study reported that EGF-induced activation of NF-κB is independent of IκB degradation and activates downstream pathways such as MEK1/2 (24). This might be due to different experimental conditions and settings. In the nucleus, EGF recruits both NF-κB subunits, p50 and p65, to its consensus sites (−116 and −538) in the EAAT1 promoter region with higher binding affinity of p65 compared with that of p50 (Fig. 4).

In the negative regulatory mechanism of EAAT1 at the transcriptional level, our findings reveal that YY1 is a critical repressor of EAAT1. Although the physiological and pathological roles of YY1 in the CNS are largely unexplored, YY1 is known to regulate several genes that play a critical role in neurological functions such as myelination in oligodendrocytes (33) and in the pathogenesis of AD in which YY1 activates β-amyloid precursor protein-converting enzyme promoter, leading to β-amyloid accumulation in the brain (65). Our findings that overexpression of YY1 decreases EAAT1 promoter activity, whereas knockdown of YY1 increases EAAT1 promoter activity, indicate that YY1 is a critical negative regulator of EAAT1 (Figs. 5 and 6). The results also suggest that YY1 plays a critical role in manganese-induced repression of EAAT1. We previously reported that manganese decreases EAAT1 mRNA/protein levels (22). The findings from the present study indicate that YY1 is a critical mediator in the inhibitory effects of manganese on EAAT1 regulation, because knockdown of YY1 reverses the manganese-induced decrease in EAAT1 promoter activity and mRNA/protein levels. A few studies on the function of YY1 on astrocytic glutamate transporters have posited that YY1 is a critical repressor of EAAT2 (34, 35) and EAAT1 (29, 30), but no study has reported the mechanism of YY1-induced repression of EAAT1 at the promoter level. Because there are multiple putative YY1 binding sites (9 sites) in the EAAT1 promoter sequences (from −1 to −1973), we knocked down YY1 expression by transducing shRNA lentiviral particles into astrocytes instead of mutating YY1 sites to determine the role of YY1 in EAAT1 regulation (Fig. 6). Notably, manganese also recruits YY1 to the YY1 consensus sites (−482 was chosen as a representative site) of the EAAT1 promoter (Fig. 7).

It is intriguing that manganese, a negative regulator of EAAT1, also activates the positive regulator NF-κB in astrocytes (19, 21). Our finding that co-expression of NF-κB p65 with YY1 completely abrogates the stimulatory effects of p65 on EAAT1 promoter activity (Fig. 8*A*) suggests that YY1 is dominant over NF-κB, overriding the positive effects of NF-κB on EAAT1 when both are activated. Interestingly, NF-κB-dependent up-regulation of YY1 by manganese or TNF-α (34, 66, 67) occurs within hours (34), whereas NF-κB-mediated positive regulation of EAAT1 by EGF requires at least 24 h (and up to several days) (20, 25). This indicates that the early activation of NF-κB by manganese could be a primary contributor to YY1 activation, which, in turn, overrides the innate NF-κB function on EAAT1 by physical interaction, leading to repression of EAAT1. This is supported by the results that manganese increased the interaction between p65 and YY1 in astrocytes (Fig. 8*B*).

In epigenetic regulation, HDACs serve as co-repressors of YY1 for the inhibition of EAAT1 promoter activity (Fig. 9). Because YY1 can act as either an activator or repressor on various genes depending on available cellular context and promoter sequences around YY1 binding sites (28, 31), interaction of YY1 with HDACs may govern the functions of YY1 on EAAT1 repression (68). HDACs regulate not only histone molecules but also non-histone proteins including YY1 via deacetylation, exerting repressive effects on the genes involved (57). This indicates that the molecular mechanisms of YY1/HDAC-induced repression of EAAT1 via deacetylation might include both histone and YY1 proteins, requiring further investigation to identify the detailed molecular mechanisms involved. Multiple HDAC isoforms from class I and II interact with YY1, leading to additional decrease of EAAT1 promoter activity, indicating that YY1 might interact with various isoforms of HDACs. HDACs also play a critical role in manganese-induced EAAT1 repression, supported by the results that HDAC inhibitors reverses manganese-induced repression of EAAT1 (Fig. 11).

Various neurodegenerative diseases linked to excitotoxicity are associated with imbalances in protein acetylation levels and transcriptional dysregulations (37, 63). Consequently, HDAC inhibitors protect against multiple neurodegenerative diseases

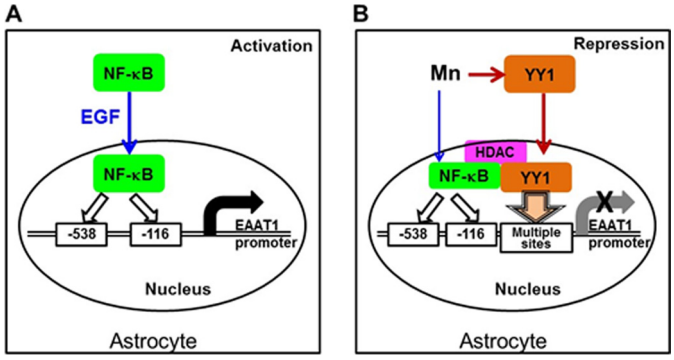

FIGURE 12. **Proposed mechanisms for the transcriptional regulation of EAAT1 via NF-κB and YY1.** *A*, for the positive regulation of the EAAT1 promoter, the canonical NF-κB pathway is activated by the stimulants such as EGF, followed by its nuclear translocation and binding to the consensus sites (−116 and −538), leading to enhancement of EAAT1 transcription. *B*, for the negative regulation of the EAAT1 promoter, YY1 is activated by the repressors such as manganese and binds to its multiple consensus sites in the EAAT1 promoter with recruitment of HDACs as epigenetic modifiers, as well as a positive regulator NF-κB, leading to repression of EAAT1.

including Huntington disease, strokes, amyotrophic lateral sclerosis, and AD (37). Further, inhibition of HDACs with VPA protects against excitotoxic neuronal death, suggesting that HDACs play an important role in glutamate-induced excitotoxic neurodegeneration (48). Dysregulation of EAAT1 is directly associated with glutamate accumulation in the synaptic clefts, leading to glutamate excitotoxicity, which is implicated in the pathophysiology of many neurodegenerative diseases (69). This underscores the important roles of HDACs in excitotoxic neurodegeneration and HDAC inhibitors as possible neuroprotectants. Our results indicating that multiple HDAC inhibitors increase the expression and function of EAAT1 and also rescue the manganese-induced reduction of EAAT1 expression and function suggest that these inhibitors could be developed as potential therapeutics against the neurological disorders associated with impairment of EAAT1. Because YY1 interacts with HDACs and NF-κB p65 and HDACs interact with NF-κB p65, it is possible that these three proteins could form a large complex to inhibit EAAT1 promoter activity. Manganese increases the physical interaction of HDACs with YY1 and p65, possibly by multiple mechanisms, including the enhancement of YY1 expression.

Taken together, we report the molecular regulatory mechanisms of EAAT1 at the transcriptional level, demonstrating that NF-κB is a critical positive regulator, and YY1 is a critical negative regulator of EAAT1. Moreover, NF-κB mediates EGF-induced up-regulation of EAAT1, whereas YY1 mediates manganese-induced down-regulation of EAAT1 (Fig. 12). HDACs are critical epigenetic co-repressors of YY1 in inhibition of EAAT1, suggesting that inhibition of YY1 and/or HDACs could be a potential molecular target to develop therapeutics against manganese neurotoxicity, as well as neurodegenerative diseases associated with excitotoxicity.

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